Characterization of clinical strains of *Aspergillus terreus* complex: molecular identification and antifungal susceptibility to azoles and amphotericin B

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Abstract

We used molecular techniques to analyse 87 (*n* = 70 patients) *Aspergillus terreus* complex isolates, all of which were identified as *A. terreus* sensu stricto. The antifungal susceptibilities determined with CLSI M38-A2 (and Etest for amphotericin B) and expressed as mg/L for range of MIC/MIC_{90}/geometric mean were as follows: itraconazole, 0.25–2/2/1.097; voriconazole, 0.125–2/2/1.176; posaconazole, 0.25–1/1/0.836; amphotericin B CLSI, 4–32/16/9.689; and Etest, 0.75–64/6/3.106. The MICs for amphotericin B were significantly higher than those found for the triazoles.

Keywords: amphotericin B, antifungal susceptibility, *Aspergillus terreus*, azoles, molecular identification

Original Submission: 20 July 2011; **Revised Submission:** 31 October 2011; **Accepted:** 1 November 2011
Editor: E. Roilides
Article published online: 7 November 2011
Clin Microbiol Infect 2012; **18:** E24–E26
10.1111/j.1469-0691.2011.03714.x

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This study was presented in part at the 21st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) and the 27th International Congress of Chemotherapy (ICC) (P-2102).

Aspergillus fumigatus is the most frequent cause of invasive aspergillosis, although other species with variable antifungal susceptibility patterns are emerging [1–3]. *Aspergillus terreus*, a species with known antifungal resistance to amphotericin B, is a clinically relevant cause of invasive aspergillosis in some hospitals [4–7].

Recent phylogenetic studies have shown that *Aspergillus* section *Terrei* includes the species *A. terreus* sensu stricto, *Aspergillus carneus*, *Aspergillus niveus*, *Aspergillus alabamensis*, and *A. terreus* var. *aureus* [8,9]. These species are morphologically indistinguishable, and can only be identified with molecular techniques. In addition, molecular identification helps us to better understand the epidemiology of invasive aspergillosis [5]. To date, with the exception of *A. niveus*, *A. terreus* sensu stricto has been the only species of the complex reported to cause invasive aspergillosis [10]. However, few studies have used molecular techniques to identify clinical isolates.

We studied 87 clinical samples (excluding otic exudates and other superficial samples) in which *A. terreus* was isolated. The samples were collected from 70 patients admitted to our institution between October 2005 and March 2010. We selected one isolate per sample. The samples were obtained from the respiratory tract (*n* = 65; 74.7%), biopsy specimens (*n* = 6; 6.9%), sterile fluids (*n* = 6; 6.9%), wounds (*n* = 6; 6.9%), and other sites (*n* = 4; 4.6%). Patients were classified according to the criteria of the European Organization for Research and Treatment of Cancer [11] as having proven invasive aspergillosis (*n* = 3), probable invasive aspergillosis (*n* = 8), or non-invasive aspergillosis.

All of the isolates were morphologically identified as *A. terreus* complex and stored. Isolates were regrown on potato dextrose agar plates. Genomic DNA of the strains was extracted from conidial suspensions with the DNeasy Tissue kit (Qiagen, Hilden, Germany), and initially treated with lyticase (Sigma-Aldrich, St Louis, MO, USA) for 2 h at 37°C. For molecular identification, we partially amplified the ITS1–5.8S–ITS2 region (primers ITS-1 and ITS-4) and the *β-tubulin* gene [12,13]. Double-stranded DNA sequencing of the products of PCR was carried out in a 3130xl analyzer (Applied Biosystems, Foster City, CA, USA). A BLAST search of all of the sequences was performed to identify the isolates. In order to investigate the presence of cryptic species, we performed a phylogenetic study including a partially amplified sequence of the ITS1–5.8S–ITS2 region and the *β-tubulin*, *enolase* and *calmodulin* genes [9]. The neighbour-joining method [14] was used to construct the phylogenetic tree based on the four regions sequenced. The data were first analysed by use of the Tamura–Nei parameter distance calculation model with gamma-distributed substitution rates

[15], and the neighbour-joining tree was constructed with MEGA version 4 [16]. A bootstrap analysis with 1000 replications was performed to determine the support for each clade. Reference sequences retrieved from GenBank were included. A clinical isolate of *Neosartorya udagawae* was included as an outgroup.

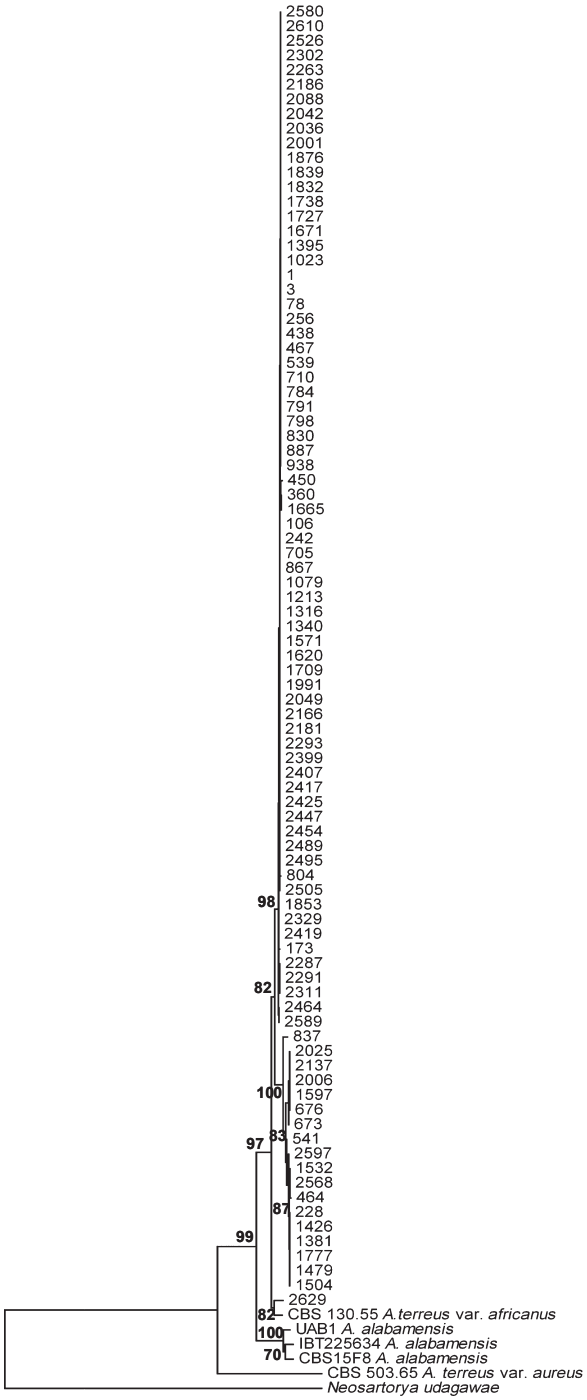

FIG. 1. Neighbour-joining tree generated from partial sequences in the ITS regions and *β-tubulin*, *enolase* and *calmodulin* genes.

Antifungal susceptibilities to itraconazole (Janssen Pharmaceutical, Madrid, Spain), voriconazole (Pfizer Pharmaceutical Group, New York, NY, USA), posaconazole (Merck Research Laboratories, Rahway, NJ, USA) and amphotericin B (Sigma, Madrid, Spain) were tested according to the CLSI M38-A2 procedure. The antifungal activity of amphotericin B was also obtained by means of the Etest (bioMérieux, Lyon, France). *A. fumigatus* (ATCC05) and *Aspergillus flavus* (ATCC04) were included as quality controls.

During the study period, we recorded 69 cases of proven/probable invasive aspergillosis with clinical isolation of *Aspergillus* species. *A. fumigatus* was found in most cases (72.4%). Although *A. terreus* was involved in 16% of the episodes, it was usually found as a co-pathogen with *A. fumigatus* or other moulds. Molecular identification with the ITS region and *β-tubulin* gene proved that all isolates were *A. terreus* sensu stricto. The phylogenetic analysis revealed that all isolates were included in the same clade (bootstrap value of 97%). A similar tree was obtained when the ITS region was removed, showing that this region yielded limited phylogenetic information. Only one of the isolates had the *A. terreus* var. *africanus* reference sequence (bootstrap value of 82%) (Fig. 1). The high bootstrap value suggests that this isolate was *A. terreus* var. *africanus*, a rarely found variety of *A. terreus* complex. This isolate was obtained from a respiratory sample of a patient with exacerbated chronic obstructive pulmonary disease, and the isolation was considered to be non-significant. The taxonomic status of *A. terreus* var. *africanus* remains unresolved, and extensive analysis on this variety is necessary [8,9].

The antifungal activities of itraconazole, voriconazole, posaconazole and amphotericin B against the 87 isolates are shown in Table 1. None of the isolates showed an MIC of >2 mg/L for itraconazole and voriconazole or >1 mg/L for posaconazole. In contrast, the MICs of amphotericin B were significantly higher than those found for the three triazoles ($p < 0.001$), regardless of the method chosen. *A. terreus* is intrinsically resistant to amphotericin B, and outcome is

TABLE 1. MICs of itraconazole, voriconazole and posaconazole against the 87 *Aspergillus terreus* isolates obtained with the CLSI M38-A2 procedure

	MIC (mg/L)			
	Range	GM	MIC_{50}	MIC_{90}
Itraconazole	0.25–2	1.087	1	2
Voriconazole	0.125–2	1.167	1	2
Posaconazole	0.250–1	0.834	1	1
Amphotericin B CLSI	4–32	9.617	8	16
Amphotericin B Etest	0.750–64	3.073	1.5	6

GM, geometric mean.

poorer in patients with invasive aspergillosis who receive antifungal treatment with this agent [17,18].

We conclude that all *A. terreus* complex isolates causing invasive aspergillosis or colonization were *A. terreus* sensu stricto. In most cases of invasive aspergillosis, *A. terreus* was considered to be a co-pathogen occurring alongside *A. fumigatus*. The isolates showed antifungal resistance to amphotericin B and remained fully susceptible to the triazoles.

Acknowledgements

We would like to thank Thomas O'Boyle for editing and proofreading the article.

This study was partially financed by grants from the Fondo de Investigación Sanitaria (FIS) PI070198 (Instituto de Salud Carlos III). J. Guinea (CP09/00055) and P. Escribano (CD09/00230) are contracted by the FIS. We are grateful to A. S. Zárate, who holds a grant from the Fondo de Investigaciones Sanitarias (Línea Instrumental Secuenciación), for her participation in the sequencing analysis. The 3130xl Genetic Analyser was partially financed by grants from Fondo de Investigaciones Sanitarias (IF01-3624 and IF08-36173).

Transparency Declaration

The authors declare no conflicts of interest.

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